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Effects of nitrogen sources on toluene degradation by *Pseudomonas putida* BZ918

Eung Sup Lee, Jae Yeon Park, Sung Ho Yeom* and Young Je Yoo†

School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Korea
*Department of Environmental and Applied Chemical Engineering,
Kangnung National University, Gangneung, Gangwon-do 210-702, Korea
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Abstract−For effective toluene degradation, the effects of a nitrogen source were studied with *Pseudomonas putida* BZ912, which was isolated from crude oil contaminated soil and is capable of degrading VOC. Two nitrogen sources, ammonia and nitrate, showed different effects on specific growth rates (0.25 hr^{-1} and 0.12 hr^{-1}, respectively), biomass yields (0.56 vs. 0.39) and specific toluene degradation rates (0.51 hr^{-1} vs. 0.26 hr^{-1}). Under the resting cell conditions, the cells pre-cultured in the ammonia-containing medium showed higher specific toluene degradation rate than that in nitrate-containing medium (0.045 hr^{-1} vs. 0.038 hr^{-1}). Ammonia as a nitrogen source was effective for degradation in high toluene concentration because high cellular biomass was accomplished. Nitrate showed slow growth rate compared to ammonia. The resting cell conditions demonstrated that it was able to degrade toluene efficiently without increasing biomass. These conditions could be a solution for degrading VOC after high cellular biomass was obtained in a biofilter. By changing the nitrogen source and the growth conditions according to the toluene concentration, the control of cell biomass and the desired removal capacity were accomplished.

Key words: Toluene Degradation, *Pseudomonas*, Ammonia, Nitrate, Biofilter, VOC

INTRODUCTION

Volatile aromatic compounds (VOCs) such as benzene, toluene and xylene (BTX) are major products of the petroleum and fine chemical industries, and among the most frequently used organic solvents [1]. However, their release to the environment is strictly controlled and they are classified as priority environmental pollutants by the U.S. Environmental Protection Agency because they are suspected to be carcinogens and can produce offensive odors [2]. They frequently enter soil, sediments and groundwater because of leakage from underground storage tanks, pipelines, accidental spills, improper practices and leaching landfills [3]. In Korea, the discharge of toluene to air is above 20% of the total amount of discharges, and a removal system of the compound urgently needs to be developed [4].

Recent efforts in developing new, efficient, and economical air pollution control technologies for VOCs treatment have focused on biological methods. For example, biofilters employed for the removal of solvent vapors have about a half or lower capital costs compared to competing processes such as incineration or adsorption on activated carbon. In addition, their operating costs are also low, in the range of 10 to 25% of those for the above-mentioned processes [5,6].

However, clogging of biofilters by growing biomass is one factor that has remarkably slowed down the implementation of biofilters on an industrial scale [7]. One of the most promising methods to prevent a biofilter from clogging is the reduction of the biomass accumulation rate and/or of the specific growth rate [8]. The challenge is to reduce biomass accumulation while maintaining a high pollutant removal rate, since growth and pollutant elimination are tightly linked. Among all nutrients, nitrogen makes up the largest fraction of dry cell mass except carbon (about 12% for a typical bacterial cell formula of $C_5H_7O_2N$) and is thus critical to sustain the cell growth and the degradation capacities of VOCs. The widespread inorganic nitrogen sources are ammonia and nitrate. If the two nitrogen sources show different kinetic values in cell growth and VOCs degradation rates, the results can be applied to biofilter operation for mitigating the clogging problem with maintaining the desirable removal capacity. With this idea, the effects of ammonia and nitrate on the degradation of toluene by the newly isolated microorganism were investigated and the resting cell conditions were introduced as an alternative strategy for the control of cell growth.

†To whom correspondence should be addressed.
E-mail: yjyoo@snu.ac.kr

MATERIALS AND METHODS

1. Microorganism and Culture Conditions

A microorganism capable of degrading toluene was isolated from oil-contaminated soil and identified as *Pseudomonas putida* BZ918. It can degrade benzene and phenol as well. The microorganism was pre-cultured at 30 °C for 24 hours in a 250 mL flask containing 100 mL of medium (100 mg/L toluene, 5.0 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 0.7 g/L KH_2PO_4, 0.7 g/L K_2HPO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$ and 200 μl trace elements). The trace elements consisted of 16.2 g/L $FeCl_3 \cdot 6H_2O$, 10.2 g/L $CaCl_3 \cdot 2H_2O$, 0.22 g/L $CoCl_2 \cdot 6H_2O$, 0.15 g/L $CuSO_4 \cdot 5H_2O$, 0.13 g/L $CrCl_3 \cdot 6H_2O$, 0.09 g/L $NiCl_2 \cdot 6H_2O$, and 40.0 g/L citric acid.

2. Toluene Degradation

After cells were harvested by centrifugation (Hitachi, SCR 18B), they were washed with distilled water several times. The cells were transferred into a 120 mL serum bottle closed with a teflon-lined septa and an aluminum crimp cap for batch degradation experiments. The serum bottle contained 0.07 g/L (OD_{660} 0.2) of microorgan-

isms and 20 mL of medium. The medium composition was 0.7 g/L K_2HPO_4, 0.7 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 200 ml trace element and various concentrations of toluene used in the experiments. The nitrogen source, either $(NH_4)_2SO_4$ or KNO_3, was supplemented to the media whose concentrations were described in figures. In experiments of the resting cell conditions, nitrogen source was not added to the culture medium. All the experiments were conducted at 30 ℃ in a shaking incubator with 200 rpm.

3. Analytical Methods

Cell concentration was measured by optical density with a spectrophotometer (UVIKON 930, KONTRON Instrument). The concentration of toluene was analyzed by head space analysis [9]. The distribution of toluene between liquid and gas phase was assumed to be in equilibrium. Controls containing no microorganism were also assayed to compensate the abiotic loss. By comparing gas phase toluene concentration in a sample bottle with that in a control bottle, the toluene consumed in a sample bottle was determined. The gas phase toluene concentration was converted to liquid phase concentration with Henry's constant. After 500 μl of head space was withdrawn by a 5 mL Hamilton gas-tight syringe, it was injected into a gas chromatograph (HP 5890) equipped with an HP-1 column and a flame-ionization detector. Helium was used as a carrier gas and GC operation conditions were 100 ℃ injection port, 150 ℃ oven, and 220 ℃ detection port temperature. The ammonia (NH_3-N) concentration was measured by Phenate method by using a spectrophotometer at 630 nm [10]. The concentration of nitrate (NO_3-N) was measured by an ion-chromatography system (Waters 432) with an IonPac Anion HR column. The mobile phase was composed of sodium borate/gluconate solution, n-butanol, acetonitrile and ultrapure water (<18 MΩ).

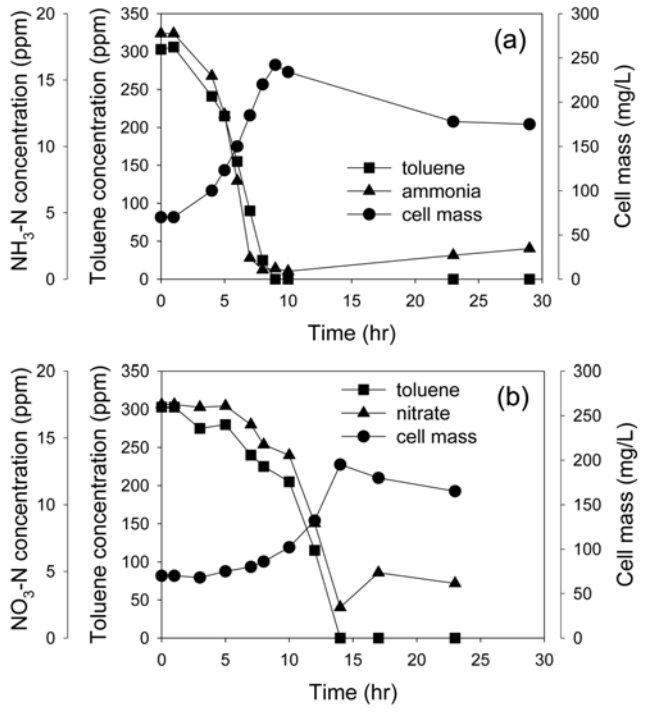

Fig. 1. Effects of nitrogen sources on the cell mass and the toluene degradation of *Pseudomonas putida* BZ918.
(a) ammonia-containing medium (b) nitrate-containing medium

RESULTS AND DISCUSSION

1. Characteristics of Toluene Degradation for Different Nitrogen Sources

The characteristics of toluene degradation by *P. putida* BZ918 were investigated for the different nitrogen sources. The profile of toluene degradation by *P. putida* BZ918 is shown in Fig. 1. When ammonia was used as a nitrogen source, 300 mg/L toluene was degraded completely within 9 hours. In the case of nitrate as a nitrogen source, it took 14 hours to degrade the same amount of toluene. The final cell mass of the former situation was around 250 mg/L, higher than the latter one by 50 mg/L. The parameters related to cell growth and toluene degradation are shown in Table 1. When ammonia was used as a nitrogen source, high growth rate and biomass yield was accomplished. A high degradation rate of toluene was also obtained. However, the high biomass yield might cause clogging in a biofilter. When nitrate was used as a nitrogen source

Table 1. Effects of nitrogen sources on specific growth rates, biomass yields, and toluene degradation rates by *Pseudomonas putida* BZ918

	Nitrogen source	
	$(NH_4)_2SO_4$	KNO_3
Specific growth rate (hr^{-1})	0.25	0.12
Biomass yield	0.56	0.39
Specific toluene degradation rate (hr^{-1})	0.51	0.26

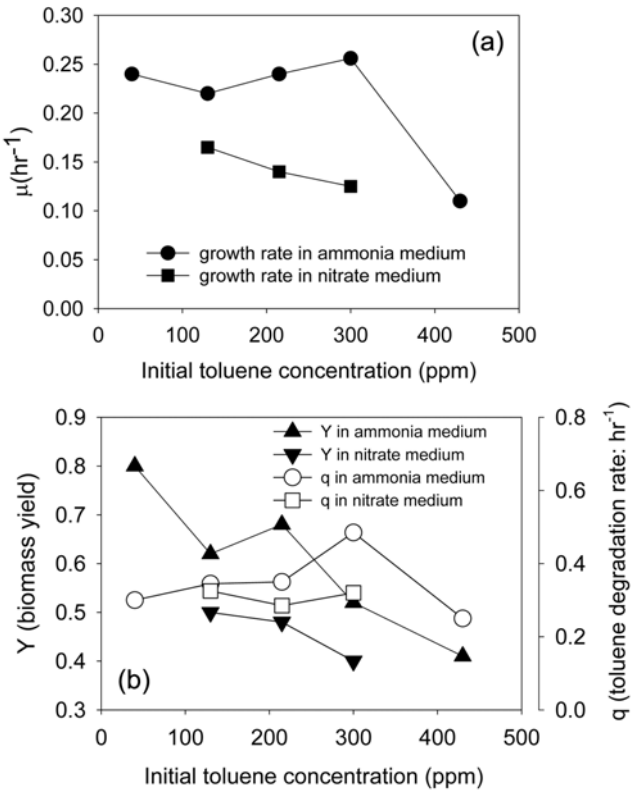

Fig. 2. Effect of toluene on the specific growth rate, the biomass yield, and the toluene degradation rate by *Pseudomonas putida* BZ918, nitrogen conc.=106 ppm.

instead of ammonia, the clogging could be repressed due to low biomass yield. Instead, lower toluene degradation rate together with low growth rate was induced. Fig. 2(a) shows the effect of toluene concentration on the grow rate of *Pseudomonas putida* BZ918. Under 300 ppm toluene, the growth rate increased when ammonia was used as a nitrogen source. When nitrate was used, instead, the growth rate decreased as toluene increased. Ammonia can be used as constituent of cells and proteins directly. On the contrary, nitrate should be converted to ammonia by the action of an assimilative nitrate reductase and an nitrite reductase in the microorganism, and this pathway makes microbes use additional energy for the reduction [11,12]. Although *Pseudomonas putida* BZ918 used phenol as a sole carbon source, this additional consumption of energy during the transformation resulted in the slow growth rate. Because toluene degradation rate is closely related with cell growth rate, those two parameters behaved similarly by the change of toluene concentration. Considering biomass yield, ammonia showed higher biomass yield in the whole range experimented (Fig. 2(b)). And both of them showed a tendency of decreasing as toluene increased, which implied that the produced energy was possibly consumed by other process such as solvent efflux pump, which removes toluene that is not metabolized by the cells [13].

When nitrogen sources were increased, similar phenomena were observed (Fig. 3). Because ammonia can be used directly, the growth rate increased as ammonia concentration increased. On the contrary, the growth rate was not affected by nitrate concentration. The transformation pathway to ammonia could be acting as a rate-limiting step in the case of nitrate, because that pathway is controlled by ammonia concentration itself. In other words, the production rate of ammonia from nitrate cannot be changed by nitrate concentration. The toluene degradation rate increased along with the growth rate when nitrogen concentration increased in the case of ammonia, and remained at the same level in the case of nitrate. The biomass yield was not affected by nitrate concentration although ammonia showed higher level of biomass yield. This implied and proved that ammonia gave fast growth and degradation rate and nitrate reduced biomass production compared to ammonia. It suggested that ammonia was required for the effective toluene degradation at high toluene concentration, but nitrate could be used as a nitrogen source at moderate toluene concentration, which probably could not lead to clogging in a biofilter. That is, the nitrogen sources, ammonia and nitrate, could be selected as a control factors in proportion to toluene concentration in a toluene degradation system in growing cell conditions.

When ammonia was supplemented during toluene degradation to the medium containing nitrate as a nitrogen source, cell growth rate and toluene degradation are shown in Fig. 4. When ammonia was added, the cell growth rate and the toluene degradation rate were increased. These results suggested a strategy of cell mass control in the operation of a biofilter by supplementing the nitrogen source intermittently.

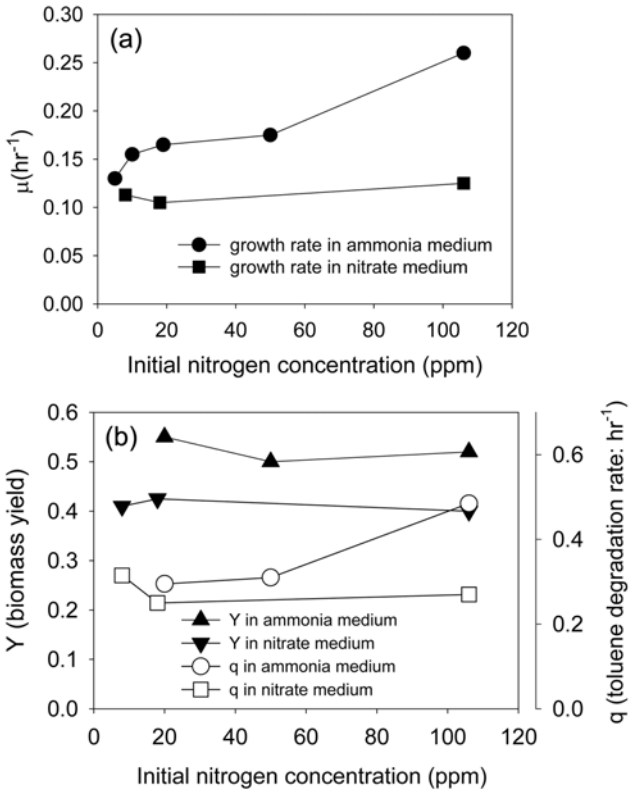

Fig. 3. Effect of nitrogen concentration on the specific growth rate, the biomass yield, and the toluene degradation rate by *Pseudomonas putida* BZ918, toluene conc.=303 ppm.

2. Characteristics of Toluene Degradation by Resting Cells

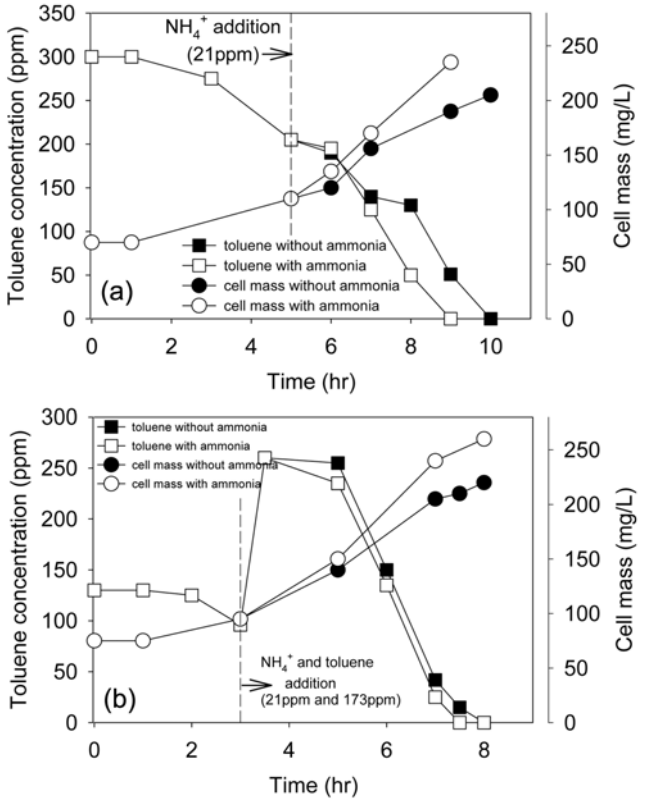

Fig. 4. Toluene degradation profiles of *Pseudomonas putida* BZ918 for the supplementation of additional toluene and nitrogen during degradation.
(a) When 21 ppm NH_4-N was added
(b) When 21 ppm NH_4-N and 173 ppm toluene were added

Table 2. Effects of various toluene concentrations and two nitrogen sources on degradation rates of toluene by resting cells of *Pseudomonas putida* BZ918

Toluene concentration (ppm)	Degradation rates of toluene	
	$(NH_4)_2SO_4$	KNO_3
130	0.0420	0.0409
217	0.0485	-
303	0.0442	0.0353
Average	0.0449	0.0381

In order to prevent clogging problems due to cell growth in a biofilter, resting cell conditions can be considered in the operation of the biofilter. The resting cell conditions in this study were established without a nitrogen source in the medium. Since one of the key substances, nitrogen here, was absent, the cells stopped growing and were less active in the degradation of toluene than those cultured in the nitrogen-containing medium. It took 11 hours and 13 hours to degrade 300 mg/L of toluene completely by the cells precultured with ammonia and nitrate, respectively. The specific toluene degradation rate by the resting cells precultured with ammonia was 0.045 hr^{-1} while that with nitrate was 0.038 hr^{-1} (Table 2). The toluene degradation rate by microorganisms precultured at ammonia-containing medium was higher. It was thought that the catechol 1,2-dioxygeanse and other related enzymes were induced more at the growth medium containing ammonia than nitrate by the reason described above. And more energy, produced and accumulated by ammonia, could give durability at high toluene concentration. The degradation rate by resting cells was lower than that by growing cells because toluene was not consumed by making cell component. Although specific degradation rates were low compared to growing cells, the time to degrade the same amount of toluene was similar to that of growing cells. The cell mass was ca. twice higher than that of growing cell conditions, but the degradation rate of this system is still high enough to degrade high concentration of toluene. The resting cell conditions could be applied to prevent increasing biomass.

3. Prediction of Toluene Degradation on Application to Biofilter

The results were applied to toluene degradation in a biofilter. Schönduve et al. [11] indicated that the relation between biomass formation and degradation was expressed in terms of the R, which is defined as

$$R=(\text{fractional inhibition of biomass formation})/(\text{fractional decrease of degradation}) \quad (1)$$

Under practical terms of operation for a biofilter, it is favorable when R is greater than 1. Schönduve et al. showed that when nitrate instead of ammonia was used as a nitrogen source, the drop in degradation of toluene was higher than the decrease in biomass formation (R=0.7). In this study, R is higher than the reported result, although the value is still under 1 (R=0.84).

The results were applied to a biofilter system model for prediction of toluene degradation. In a biofilter, the toluene degradation rate can be indicated as follows:

$$q=\frac{\Delta T}{X_{avg}\cdot\Delta t}=\frac{C_{in}-C_{out}}{X\cdot EBRT}=\frac{EC}{X} \quad (2)$$

$$EBRT=\frac{V}{Q} \quad (3)$$

$$EC=\frac{Q}{V}\cdot(C_{in}-C_{out}) \quad (4)$$

Where X is the cell mass which is attached to the packing materials per reactor volume (mg/L); C_{in}, the influent toluene concentration (mg/L); C_{out}, the effluent toluene concentration (mg/L); EBRT, empty bed retention time (hr); EC, elimination capacity of toluene (mg/L/hr); V, bed volume of biofilter (L); Q, toluene flow rate (L/hr).

The cell mass (X) attached to the polyurethane coated by activated carbon was measured separately and turned out to be 387 mg/

Table 3. Degradation parameters in a biofilter for growing cells and resting cells

Parameters	Growing cells		Resting cells	
	NH_4-N medium	NO_3-N medium	NH_4-N medium	NO_3-N medium
q	0.350	0.289	0.0449	0.0381
Y*	0.570	0.396	0	0
R	0.84			
$EC_{predict}$ ($g/m^3/h$)	135	112	17.4	14.7

*Initial toluene concentration=303 ppm.
EC: Elimination capacity.

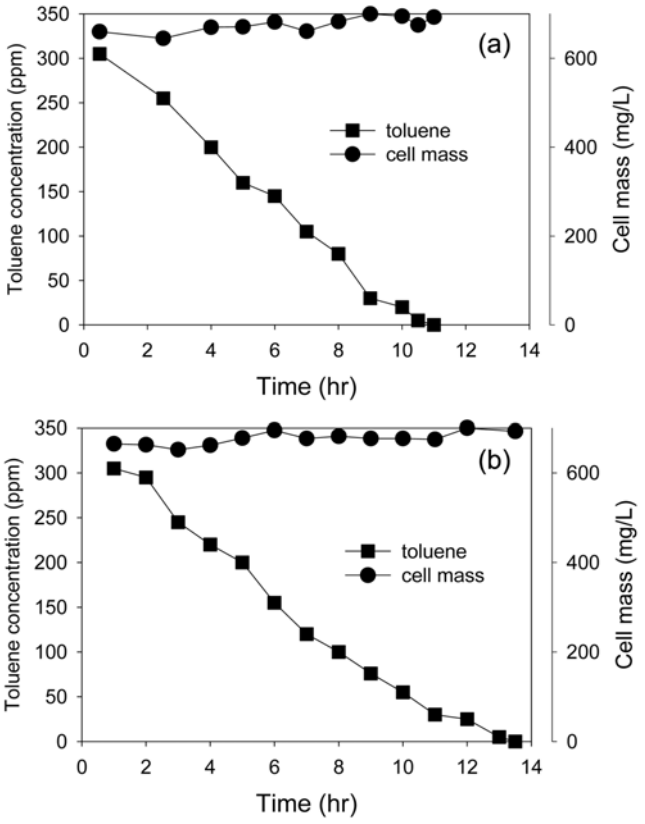

Fig. 5. Toluene degradation profiles by resting cells.
(a) precultured in the ammonia-containing medium
(b) precultured in the nitrate-containing medium

L. The predicted values of EC are shown in Table 3. The predicted EC by growing cells was higher than the predicted EC by resting cells. In the previous studies on the toluene degradation in a biofilter, the elimination capacities ranged from 25 °C to 165 $g/m^3 \cdot h$ [13,15,16]. The EC values for growing cell conditions were in high positions, but those for resting cell conditions were not. The reason might be low X value. Yeom showed that X was assumed and measured to be from 5,000 to 7,593 mg/L, which is 20 times more than this experiment [9,17]. If biocompatible adsorbent which had a high adsorption capacity for cells was used, the EC values would increase. However, both EC values for ammonia and nitrate showed compatible values even under insufficient biomass attached.

The mechanism of biofilm formation is a complicated process whose subject is still being studied by many scientists [18]. Although many factors such as osmolarity, quorum sensing, catabolic repression, starvation *etc.* are influencing the biofilm formation, growth factors revealed in this study could affect the biofilm formation due to their direct effects on planktonic state of microbes and the microbial growth on the surface of biofilm. Therefore, the strategies suggested in this study might be a good solution to control biomass in a biofilter.

CONCLUSIONS

Since the two nitrogen sources showed remarkably different effects on cell growth and toluene degradation, the duration of a biofilter could be lengthened with a desirable elimination capacity by switching the two nitrogen sources according to the concentration of influent toluene during operations. And the resting cell conditions suggested an idea of controlling biomass in the biofilter while maintaining the capacity of the biofilter. The demonstration of the idea in the biofilter remains for further study.

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